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Mediator is a transducer of amyloid-precursor-protein-dependent nuclear signalling

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Regulated intramembrane proteolysis of the amyloid precursor-protein (APP) produces both a characterstic amyloid-β peptide that contributes to neuritic plaque formation and neurodegeneration in Alzheimer disease and a small APP intracellular domain (AICD) that transcriptionally activates genes implicated in Alzheimer disease pathology. Although the biochemical events leading to amyloidogenic APP processing at the cell membrane have been described in detail, comparably little is known about the mechanistic basis of AICD-dependent gene regulation in the nucleus. In this study, we show that the AICD activates transcription by targeting MED12, an RNA polymerase II transcriptional Mediator subunit that is implicated in human cognitive development. The AICD binds to MED12/Mediator *in vitro* and *in vivo*. Disruption of the AICD/MED12 interaction inhibits AICD transactivation potential and expression of AICD target genes. Mediator, in a MED12-dependent manner, occupies only AICD-bound promoter DNA, indicating that the AICD recruits Mediator to activate transcription. These results identify the MED12 interface in Mediator as a crucial transducer of AICD transactivation and a potential therapeutic target in Alzheimer disease.

Keywords: Alzheimer disease; amyloid precursor protein; MED12; Mediator

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INTRODUCTION

The amyloid precursor protein (APP) and two APP-like proteins—APLP1 and APLP2—comprise a family of type I integral membrane proteins with essential and overlapping roles in brain development and function (Zheng & Koo, 2006; Walsh *et al*, 2007). Among its family members, APP is notable for its association with Alzheimer disease, a progressive neurodegenerative disorder characterized by early short-term memory deficits and eventual loss of cognitive and executive functions. The pathological characteristics of Alzheimer disease are the presence of extracellular neuritic plaques enriched in amyloid-β peptides and intracellular neurofibrillary tangles comprising hyperphosphorylated tau protein (Mucke, 2009). Amyloid-β production through amyloidogenic processing of APP by membrane-bound β- and γ-secretases results in the simultaneous production of a 50-amino-acid carboxy-terminal fragment corresponding to the APP intracellular domain (AICD; Haass, 2004). APLP1 and APLP2 are proteolytically processed in a mechanistically similar way, although sequence divergence within their amyloid-β regions precludes plaque formation (Scheinfeld *et al*, 2002; Li & Sudhof, 2004). Notably, the AICD is among the most highly conserved protein regions found in APP family members, suggesting that it is functionally important.

The AICD has been reported to translocate into the nucleus and activate gene transcription (Cao & Sudhof, 2001; Gao & Pimplikar, 2001). Notably, several AICD-target genes have been implicated in cellular processes relevant to Alzheimer disease. For example, neprilysin—a synaptic ectoenzyme that degrades amyloid-β in the extracellular space—helps to maintain the concentration of amyloid-β below the threshold for self-aggregation, whereas p53-mediated apoptosis has been implicated in Alzheimer-disease-associated neuronal death (Pardossi-Piquard *et al*, 2005; Alves da Costa *et al*, 2006). More recently, a cohort of AICD-target genes implicated in control of cytoskeletal dynamics, including α2-actin, IGFBP3 and TAGLN, was found to be upregulated in the frontal cortex of Alzheimer disease patients, whereas another AICD-regulated gene, *Aquaporin 1*, was shown to be upregulated in astrocytes surrounding amyloid-β plaques within the brains of Alzheimer disease patients (Muller *et al*, 2007; Huysseune *et al*, 2009). Together, these observations suggest that AICD-dependent nuclear signalling might contribute to the pathophysiology of Alzheimer disease.

Little is known about the mechanism by which the AICD activates gene transcription in the nucleus. Established interactions between the AICD, the transcriptional adaptor Fe65 and acetyltransferase Tip60 suggest it is linked to histone modification (Cao & Sudhof, 2001), which is supported by work documenting AICD-dependent histone acetylation on the *neprilysin* promoter

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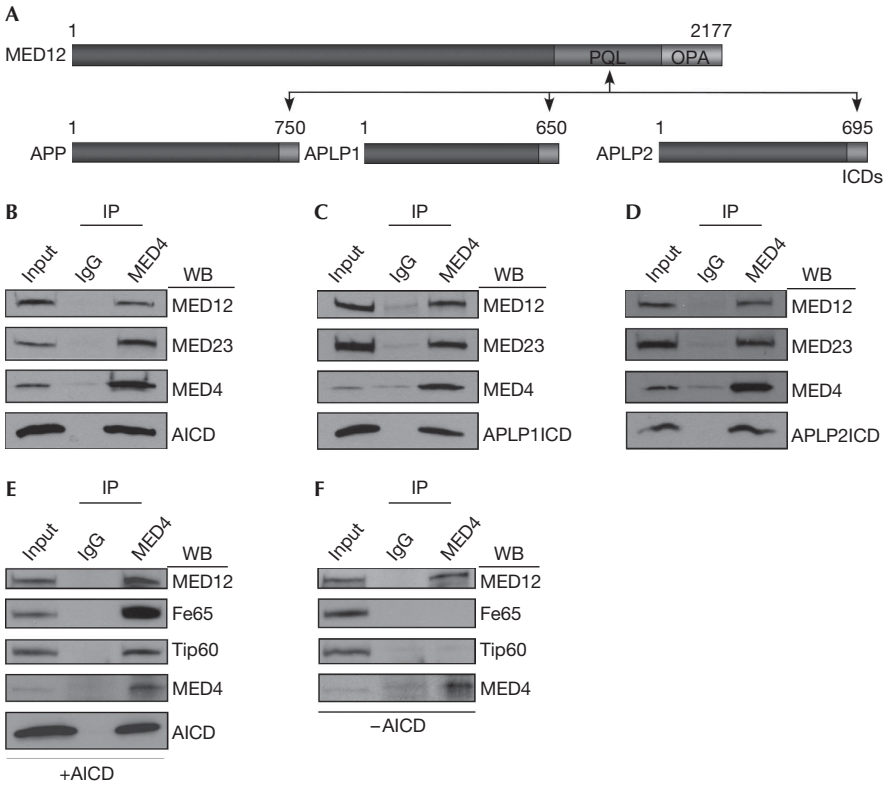

Fig 1 | Amyloid precursor protein (APP)/APP-like protein intracellular domains (APLP ICDs) bind to MED12/Mediator. (**A**) Schematic summary of reciprocal binding domains on APP/APLP proteins and MED12. A proline-, glutamine-, leucine-rich (PQL) domain on MED12 interacts with all three APP/APLP ICDs. Numbers refer to amino-acid coordinates. (**B–F**) Nuclear extracts from HeLa cells transiently expressing the ICD50 of (**B**) APP, (**C**) APLP1, (**D**) APLP2 or (**E**) Fe65/Tip60 with or (**F**) without APP ICD were immunoprecipitated with IgG or antibodies for the core Mediator subunit MED4. Immunoprecipitates were resolved by SDS–PAGE and processed by western blot analysis using the specified antibodies. Input, 10% of nuclear extract used for immunoprecipitation. AICD, amyloid precursor protein intracellular domain; APLP, amyloid precursor protein-like protein; APP, amyloid precursor protein; ICD, intracellular domain; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; PQL, proline-, glutamine-, leucine-rich; WB, western blot.

(Belyaev *et al*, 2009). However, a physical or functional AICD target within the RNA polymerase II general transcription machinery suggesting a direct mechanistic basis for gene regulation has not been reported so far. In this paper, we identify the MED12 subunit within the RNA polymerase II transcriptional Mediator as a direct physical target and functional transducer of the AICD, as well as the intracellular domains of APLP1 and APLP2. Mediator is a conserved multi-subunit signal processor through which regulatory information conveyed by gene-specific transcription factors is transduced to RNA polymerase II in order to alter gene expression programmes controlling cell growth, homeostasis, development and differentiation (Kornberg, 2005; Malik & Roeder, 2005). MED12 is implicated in vertebrate neuronal development, and genetic variation in MED12 is associated with cognitive and behavioural dysfunction in humans (Philibert *et al*, 2001; Wang *et al*, 2006; Risheg *et al*, 2007; Schwartz *et al*, 2007). Our findings that MED12 mediates the AICD transactivation potential and expression of AICD-target genes identify MED12 as a new component in the APP-dependent nuclear-signalling pathway, and further implicate Mediator in a range of developmental and pathological processes driven by APP signal transduction.

RESULTS

APP/APLP ICDs bind to MED12/Mediator

To clarify the role of MED12 in neural-specific transcription control, we processed the MED12 C-terminus (MED12C; amino acids 1,616–2,177) through a yeast two-hybrid screen using a human foetal brain cDNA library (Zhou *et al*, 2006) and recovered APLP1, a neural-restricted APP-family member (Lorent *et al*, 1995). To confirm a physical interaction between MED12 and APLP1 and map their reciprocal binding domains, we tested the ability of glutathione *S*-transferase (GST)–MED12 and GST–APLP1 derivatives to bind to a panel of APLP1 and MED12 truncation fragments, respectively. This analysis identified the principal reciprocal interaction surfaces on each protein which include a proline-, glutamine- and leucine-rich (PQL) domain on MED12 (amino acids 1,616–2,050) and the C-terminal 50 amino acids of APLP1 corresponding to its transcriptionally active intracellular domain (ICD50; Fig 1A; supplementary Figs S1 and S2 online). As the ICDs of all three APP family members are highly conserved, we tested and confirmed that MED12 could also bind to APP and APLP2 ICD50 (Fig 1A; supplementary Figs S1 and S2 online). Thus, MED12 binds specifically through its PQL domain

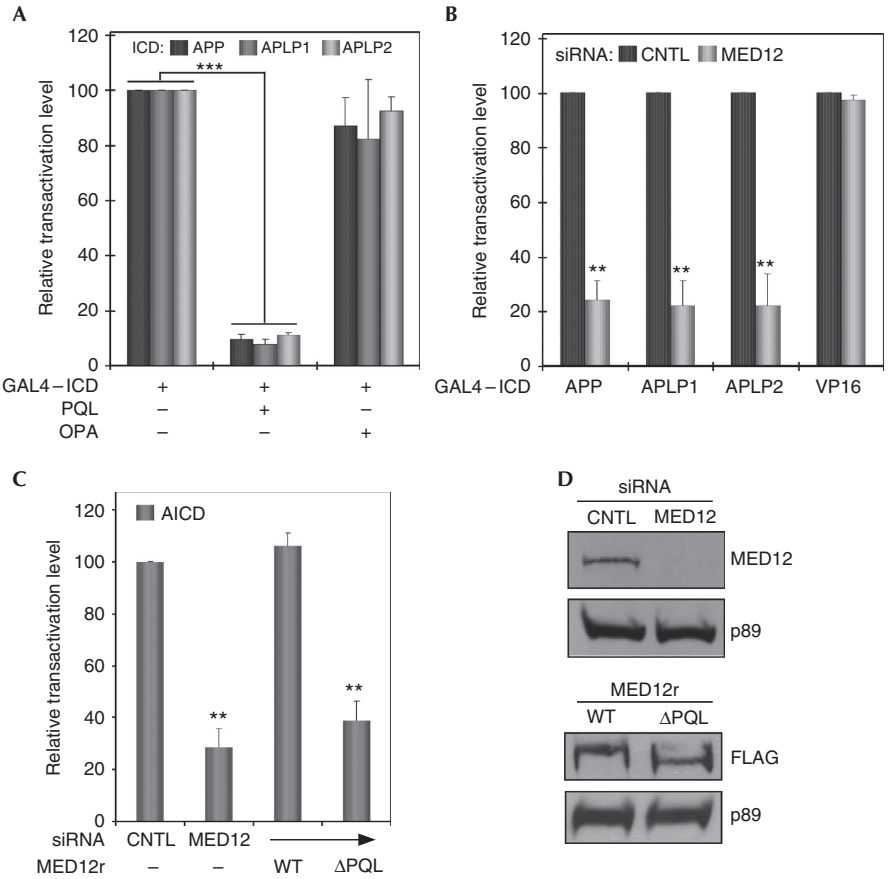

Fig 2 | Amyloid precursor protein (APP)/APP-like protein intracellular domains (APLP ICDs) functionally interact with MED12. (**A–C**) HeLa cells were transfected with GAL4–APP/APLP ICDs or GAL4–VP16 with either (**A**) MED12 proline-, glutamine-, leucine-rich (PQL) or OPA domains or (**C**) siRNA-resistant FLAG-tagged MED12 wild-type or ΔPQL derivatives. (**B,C**) Cells were transfected with control (CNTL) or MED12-specific siRNAs 48 h before plasmid transfections. Cells collected 36 h after plasmid transfections were processed for luciferase and β-gal activities. Normalized luciferase activities associated with individual GAL4 derivatives were calculated relative to luciferase activities obtained in cells transfected with (**A**) GAL4 alone or (**B,C**) GAL4 and CNTL siRNA. For comparison, the relative luciferase activity for each GAL4 derivative was assigned a transactivation level of 100%; its corresponding activity in the presence of (**A**) the PQL or OPA domains or (**B,C**) MED12-specific siRNA is expressed relative to this value. Data represent the mean ± s.e.m. of at least three independent transfections performed in duplicate. Except where specified (**A**), asterisks denote statistically significant differences relative to CNTL siRNA (Student's *t*-test $^{**}P<0.005$; $^{***}P<0.001$). (**D**) Western blots from representative transient knockdown/rescue assays using the specified antibodies (TFIIH p89 subunit acts as a loading control). AICD, amyloid precursor protein intracellular domain; APLP, amyloid precursor protein-like protein; APP, amyloid precursor protein; CNTL, control; ICD, intracellular domain; PQL, proline-, glutamine-, leucine-rich; siRNA, short-interfering RNA, WT, wild type.

to the transcriptionally active ICDs of all three APP/APLP family members.

To determine whether APP/APLP ICDs interact with intact Mediator, we immunoprecipitated Mediator from nuclear extracts of HeLa cells transiently expressing APP/APLP ICDs and examined the immunoprecipitates for the presence of each ICD by western blot assay. This analysis revealed specific coimmunoprecipitation of each APP/APLP ICD50 with intact Mediator, confirming their association in mammalian cells (Fig 1B–D). Notably, Fe65 and Tip60—established co-activators of the AICD—were coprecipitated with Mediator in the presence, but not the absence, of coexpressed AICD, demonstrating that Fe65 and Tip60 can form an AICD-dependent multimeric complex with Mediator in mammalian cells (Fig 1E,F).

APP/APLP ICDs functionally interact with MED12

If the APP/APLP ICDs functionally target the MED12 interface in Mediator, their corresponding binding (PQL) domain on MED12 might inhibit their respective transactivation potentials through dominant-negative interference. To test this possibility, we examined the influence of MED12–PQL expression on the transactivation activity of each APP/APLP ICD tethered to the GAL4 DNA-binding domain (Cao & Sudhof, 2001). Ectopic expression of the MED12–PQL domain, but not of its neighbouring MED12–OPA domain, to which APP/APLP ICDs do not bind (supplementary Fig S2 online; data not shown), efficiently inhibited the transcriptional activities of all three APP family ICDs (Fig 2A).

To further examine the functional interaction between APP/APLP ICDs and MED12/Mediator, we monitored the influence of

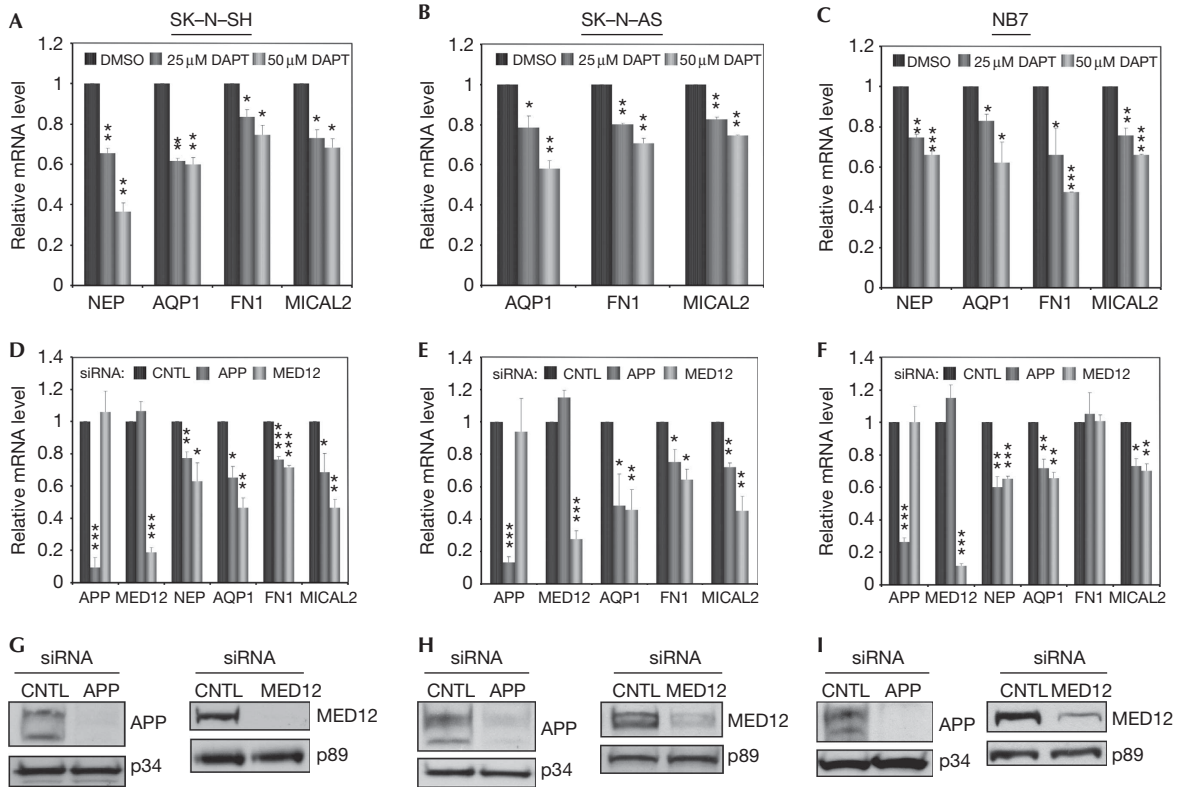

Fig 3 | MED12 mediates amyloid precursor protein intracellular domain (AICD)-dependent target gene expression. (**A–C**) RNA from SK–N–SH, SK–N–AS or NB7 cells treated with 0, 25 or 50 μM DAPT or (**D–F**) control (CNTL), APP- or MED12-specific siRNAs was subjected to RT–qPCR. mRNA levels for the indicated genes were normalized to RPL19 mRNA and expressed relative to their corresponding mRNA levels in (**A–C**) DMSO-treated or (**D–F**) control siRNA-treated cells. Data represent the mean ± s.e.m. of at least three independent experiments performed in duplicate. Asterisks denote statistically significant differences relative to (**A–C**) DMSO or (**D–F**) CNTL siRNA (Student's *t*-test $*P<0.05$; $**P<0.005$; $***P<0.001$). Note that *NEP* expression in SK–N–AS cells was below the limit of reliable detection and therefore omitted from analysis. (**G–I**) Western blots from representative transient knockdown assays using the specified antibodies (TFIIE p34 and TFIIH p89 subunits serve as loading controls). APP, amyloid precursor protein; CNTL, control; DMSO, dimethylsulphoxide; NEP, neprilysin; RT–qPCR, reverse transcription–quantitative PCR; siRNA, short-interfering RNA.

RNA interference-mediated MED12 suppression on the transactivation activity of the ICD of each APP family member. Depletion of MED12 strongly inhibited the transcriptional activity of each APP/APLP ICD (Fig 2B,D). By contrast, depletion of MED12 had no influence on the transactivational activity of the herpes simplex virus VP16 transactivation domain (Fig 2B), the established targets of which in Mediator are MED17/25 (Ito *et al*, 1999). Thus, the influence of MED12 knockdown in this context seems to be restricted to the APP-family ICDs. To confirm that MED12 mediates transactivation by the AICD through direct interaction, we assessed the respective abilities of short-interfering RNA (siRNA)-resistant wild-type MED12 (MED12r) and a siRNA-resistant AICD–binding-defective MED12 derivative lacking the PQL domain (MED12r–ΔPQL) to rescue the AICD transactivation in MED12-depleted cells. When expressed at equivalent levels, MED12r, but not MED12r–ΔPQL, elicited efficient rescue of AICD-directed transactivation (Fig 2C,D). This finding precludes the possibility that reduced AICD-driven transactivation elicited by MED12 siRNA derives from a nonspecific 'off-target' effect, and confirms that the AICD physically interacts with the MED12 interface in Mediator to activate transcription.

MED12 mediates AICD-dependent gene expression

To examine the role of MED12/Mediator as a transducer of AICD-dependent nuclear signalling in a more physiological context, we monitored the effect of MED12 depletion on the expression levels of AICD-regulated genes in three different human neuroblastoma cell lines: SK–N–SH (Tachida *et al*, 2008), SK–N–AS (Tillement *et al*, 2006) and NB7 (Belyaev *et al*, 2009). Of those genes identified as targets of AICD-dependent regulation from previous functional studies (Pardossi-Piquard *et al*, 2005; Alves da Costa *et al*, 2006; Muller *et al*, 2007), we confirmed four as targets of presenilin- and AICD-dependent regulation in neuroblastoma cells (*Neprilysin* (*NEP*), *Aquaporin 1*, *Microtubule-associated monoxygenase, calponin and LIM domain containing 2* (*MICAL2*) and *Fibronectin 1*). Thus, pharmacological inhibition of γ-secretase with DAPT (Fig 3A–C) or RNAi-mediated APP depletion (Fig 3D–I) significantly reduced the expression levels of *NEP*, *Aquaporin 1*, *MICAL2* and *Fibronectin 1* in at least two out of the three neuroblastoma cell lines examined. Notably, RNAi-mediated MED12 depletion also significantly reduced expression of these four genes in a manner perfectly concordant with their regulation by the AICD, implicating MED12/Mediator in

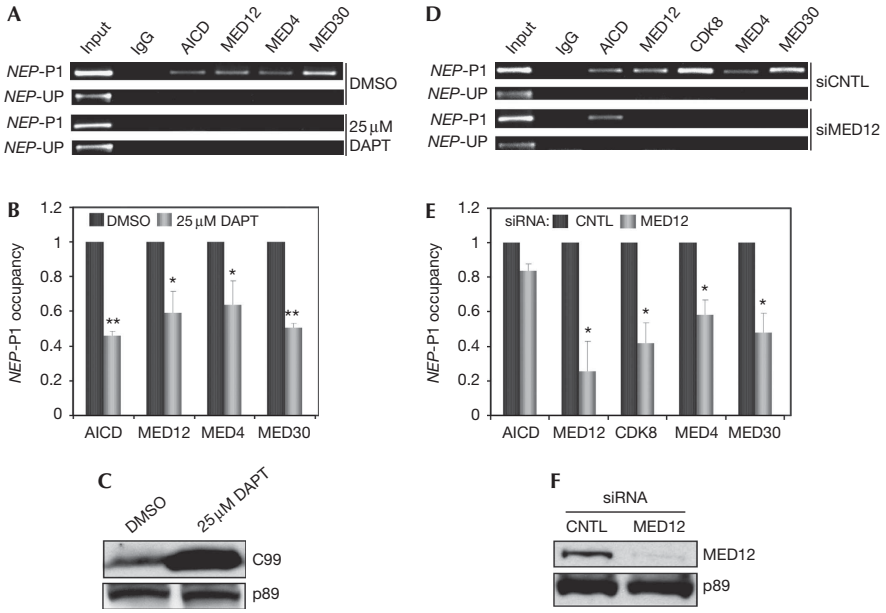

Fig 4 | Mediator is recruited to the amyloid precursor protein intracellular domain-bound *NEP* promoter in a MED12-dependent manner. Soluble chromatin from NB7 cells treated with (**A**,**B**) 0 or 25 μM DAPT or (**D**,**E**) control or MED12-specific siRNA was subjected to immunoprecipitation using the indicated antibodies. Immunoprecipitated chromatin was analysed by (**A**,**D**) semiquantitative or (**B**,**E**) quantitative PCR using primers specific for *NEP* promoter 1 (*NEP*-P1) or upstream (*NEP*-UP) sequences. In (**B**,**E**) data represent the mean ± s.e.m. of at least three independent experiments performed in triplicate. Asterisks denote statistically significant differences in *NEP*-P1 occupancy for each protein, relative to its occupancy in either (**B**) DMSO-treated or (**E**) CNTL siRNA-treated cells. (Student's *t*-test $*P<0.05$; $**P<0.005$). (**C**,**F**) Western blots from representative enzyme inhibition and transient knockdown ChIP assays using the specified antibodies. AICD, amyloid precursor protein intracellular domain; C99, amyloid precursor protein carboxy-terminal fragment and γ-secretase substrate; ChIP, chromatin immunoprecipitation; CNTL, control; DMSO, dimethylsulphoxide; NEP, neprilysin; siRNA, short-interfering RNA.

AICD-dependent gene regulation (Fig 3D–I). Finally, of these four genes only *MICAL2* was coordinately regulated similarly in HeLa cells (data not shown), suggesting possible neural-cell-type-specific gene regulation by the AICD and MED12/Mediator.

To explore the mechanistic relationship between the AICD and MED12/Mediator, we used chromatin immunoprecipitation (ChIP) to monitor their interdependence for occupancy of the *NEP* promoter. Depletion of AICD levels in NB7 cells through DAPT inhibition of γ-secretase activity significantly reduced *NEP* promoter occupancy by the AICD, as expected, as well as by MED12/Mediator (Fig 4A–C). By contrast, RNAi-mediated MED12 depletion reduced occupancy of the *NEP* promoter by Mediator, but not by the AICD, suggesting that the AICD recruits Mediator to the *NEP* promoter in a MED12-dependent manner (Fig 4D–F).

DISCUSSION

Despite increasing evidence for support a nuclear function for the AICD in gene regulation, none of the more than 20 previously reported AICD interaction partners represent a direct link to the RNA polymerase II general transcription apparatus. It has thus been difficult to determine how the AICD might activate transcription. Our identification of MED12/Mediator as a direct physical and functional target of the AICD suggests a simple solution to this problem. We propose a model in which promoter-bound AICD recruits Mediator through its MED12 interface to stimulate the assembly, activation and/or regeneration of transcription complexes on its target genes (Malik & Roeder, 2005). Alternatively, or additionally, Mediator might facilitate the imposition of an AICD-driven transcriptionally permissive chromatin environment (Ding *et al*, 2008).

Similarly to APP, MED12 is expressed in both developing and adult vertebrate tissues (Rocha *et al*, 2010). Nevertheless, early developmental phenotypes of MED12 knockout mice support a gene-specific, as opposed to a ubiquitous, function for MED12 in RNA polymerase II transcription. For example, although MED12-deficient mouse embryos die before completing gastrulation, they nevertheless develop further than those lacking the core Mediator subunits required for RNA polymerase II activity (Tudor *et al*, 1999; Rocha *et al*, 2010). Furthermore, MED12 hypomorphic mutant embryos support unperturbed development of many embryonic structures, processes and marker genes (Rocha *et al*, 2010). The broad distribution of MED12 might therefore endow Mediator with a unique interface through which it might be selectively recruited by diverse gene-specific transcription factors, including the AICD.

Notably, among the 33 subunits that comprise Mediator, MED12 is uniquely implicated in vertebrate neural development and disease. In zebrafish, MED12 has been shown to be required for proper development of the brain and neural crest, among other organs, where it is important in the production of monoaminergic neurons and cranial sensory ganglia (Wang *et al*, 2006). In humans, polymorphisms in the *Xq13* gene encoding MED12 have

been linked with neuropsychiatric illnesses including schizophrenia and pyschosis, and MED12 mutations have been shown to cause two X-linked mental retardation disorders: FG syndrome and Lujan syndrome (Philibert *et al*, 2001; Risheg *et al*, 2007; Schwartz *et al*, 2007). Our identification of MED12 as a direct ingress in Mediator for AICD-dependent nuclear signalling suggests a possible link between the biological function of APP in brain development and disease and its role in transcriptional regulation. Finally, because genetic variation in MED12 is causally linked with cognitive and behavioural dysfunction in humans, it will be interesting to determine whether polymorphisms in *MED12* might also predispose certain individuals to Alzheimer disease.

METHODS

Antibodies. CDK8 (sc-1521), TFIIHp89 (sc-293), TFIIEβ (sc-238), Santa Cruz Biotechnology; MED12 (A300-774A), Bethyl Laboratories; MED23 (551175), BD pharmingen; FLAG (F3165), Sigma; APP/APLP1/APLP2 (171610/171615/171616), Calbiochem/EMD Biosciences. Rabbit MED4 polyclonal antibodies were produced and purified as described (Kim *et al*, 2006).

Cell culture. SK–N–SH and SK–N–AS cell lines were kindly provided by Drs Veronica Galvan and Luis Penalva (University of Texas Health Science Center at San Antonio, Texas, USA), respectively, and cultured in DMEM, 10% FBS (Hyclone). NB7 cells, a gift from Dr Jill Lahti (St Jude Children's Research Hospital, Memphis, Tennessee, USA) were cultured as described (Belyaev *et al*, 2009). HeLa cells were cultured as described (Kim *et al*, 2006).

Plasmids. pMst-AICD, pMst-APLP1ICD and pMst-APLP2ICD for transient reporter assays were generated by PCR-based subcloning of APP/APLP ICD-encoding cDNA sequences into the pMst vector (from Dr Thomas Südhof). pCMV5-Fe65 for transient reporter assays, pM-Fe65 and pM-hTip60 for immunoprecipitations were gifts from Dr Thomas Südhof. Plasmids pGAL4-VP16, pG_5E1B-luc, pACT-β-galactosidase and p3xFLAG-CMV-hMED12r wild type and ΔPQL for transient reporter assays have been described (Kim *et al*, 2006; Zhou *et al*, 2006; Ding *et al*, 2008).

Transfection, reporter assays and RNAi. Cells were transfected with plasmid DNA using FuGENE 6 (Roche Applied Science); activities of luciferase (Promega Corporation) and β-galactosidase (Applied Biosystems) were determined as described previously (Zhou *et al*, 2006; Ding *et al*, 2008). For RNAi, cells were transfected with MED12-specific (M-009092-00), APP-specific (M-003731-00) or control (D-001210-01-05) siRNAs (Dharmacon; 20–40 nM) using Transit SiQuest (Mirus Bio Corporation).

Quantitative real-time reverse transcription–PCR. RNA extracted from cells 48 h after addition of DAPT (Calbiochem/EMD Biosciences) or 72 h after siRNA transfections was reverse-transcribed using oligo (dT) and superscript iii (invitrogen). Quantitative PCR was performed using a 7900HT fast real-time PCR system (Applied Biosystems) and Absolute SYBR Green ROX mix (ABgene). For each sample, the amplification plot and the corresponding dissociation curves were examined. Primer sequences are available on request.

Immunoprecipitations and ChIP. HeLa cells were collected 48 h after transfection with or without $pCS3+H_6T_7$-ICDs (APP/APLP), pM-Fe65 and pM-Tip60. Nuclear extracts—prepared as described previously (Ding *et al*, 2008)—were adjusted using 200 mM KCl and 0.2% NP-40, followed by incubation for 12 h at 4 °C with 3 μg of either MED4-specific rabbit polyclonal antibody or rabbit IgG. Immune complexes recovered by incubation with protein A-sepharose (20 μl) for 2 h at 4 °C were washed five times for 5 min each with 0.3-M KCl D buffer (0.2% NP-40), eluted in Laemmli sample buffer and resolved on 4–20% tris–glycine gradient gels (Lonza) for immunoblot analysis. Chromatin immunoprecipitation was performed as described previously (Ding *et al*, 2008) from NB7 cells treated with 0 or 25-μM DAPT or control, APP, or MED12-specific siRNAs using 3 μg of control or specific antibodies. Purified DNA was analysed by conventional or real-time PCR. Primer sequences have been described previously (Belyaev *et al*, 2009).

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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